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ABSTRACT

Described was research on the behavioral and learning effects of lead poisoning or malnutrition in rats. It is explained that approximately 200 rats (either weanling, adult, pregnant, or nursing) were injected with various amounts of lead. It was found that symtomatic levels of lead in weanling or adult rats produced no obvious behavioral or learning impairments, though asymptomatic doses of lead produced a 100% abortion rate in pregnant rats and retarded development and learning impairments in the offspring of lactating rats. The author describes a completely automated closed-field maze series for rats developed to counter inaccuracies among observers using a conventional maze. It is reported that experiments with 130 rats to determine the cause of pica (the voluntary ingestion of lead by children) showed that calcium-deficient rats ingested significantly more lead than non-deficient or iron-deficient rats. Experiments attempting to produce picas in adult rats by conditioning procedures were said to have negative results. The most severe effects of protein malnutrition on learning ability were found to occur when the malnutrition was imposed prenatally. (DB)

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Final Report

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Research relating to the learning of children identified as having experienced malnutrition and/or heavy metal poisoning.

October 15, 1973

United States Department of Health, Education, and Welfare
Office of Education
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Chapter 1
Introduction

The work performed under this grant was concerned with the examination of nutritional and environmental variables that might influence educational performance. The major focus of this report is on lead as a material that can influence learning performance. It has been estimat-d that a quarter of a million children each year voluntarily ingest enough lead to produce severe lead poisoning. A concomitant of the physical symptoms of lead poisoning is an irreversible retardation. In recent years it has become apparent that not all cases of lead poisoning result from the child's voluntary ingestion of lead -- pica -- but that lead is absrobed in large amounts in children living near freeways or industrial plants that have leaded byproducts.

There was a dearth of experimental data on the behavioral effects of lead when this research began. Only two studies, Brown et al. (1971) and Bullock et al. (1966), had examined the effects of lead on learning ability in rats, both with negative findings. There has been no study experimentally trying to determine why children engage in voluntary lead ingestion - pica. Chapter 2 presents our experiments on the effects of lead injections on the learning ability of rats. We injected various doses into adult weanling, nursing and pregnant rats and tested them on a Hebb-Williams maze test, a series of twelve different problems. This testing sequence was shown by Davenport and Dorcey (1971) to be the most sensitive indicator of learning deficits in rats. Thus subtle differences would be more apparent than with other means of testing.

We readily discovered problems with the use of the Hebb-Williams maze series. It requires an observer to be present and watching the animal as it moves through the maze. We found with our observers a ready fatigue which limited the number of animals that could be tested at one time, and that often the data taken by an observer were not accurate. In response to these problems we have devised a completely automated Hebb-Williams maze that reliably scores the animal's performance automatically. Since the maze series does represen a highly sensitive measure of learning deficits, the development of a completely automated maze should facilitate the study of a wide range of experimental variables as they affect learning ability. The description and validation of the maze is presented in Cahpter 3.

Chapter 4 examines the problem of lead pica--why should children voluntarily ingest lead when it produces painful symptoms? Any other organism that has been studied seems to avoid the ingestion of dangerous substances. Lead solutions were shown not to be preferred by normal rats, nor did rats seem to be able to detect increased levels of lead injected into them. But when rats were deprived of calcium they showed a significant increase in both the total amount of lead ingested and in the proportion of lead to water ingested. Rats with iron deficiency failed to increase lead ingestion.

Chapter 5 describes some additional studies on lead pica trying to generalize the results to adult rats which proved not possible and examining a proposed model for why children do ingest lead.

Chapter 6 provides data obtained in a related series of experiments on the effects of protein malnutrition on learning ability. Rats were exposed to one of three diets with differing amounts of protein at one of three developmental states--prenatally, postnatally or post-weaning. At a later age the rats were tested on the learning problems of the Hebb-Williams maze. The results indicated that greater degrees of protein malnutrition and earlier stages of administration produced the greatest degree of learning disability.

Chapter 2
Learning deficits in lead injected rats

The use of laboratory animals such as the rat as experimental models for the behavioral effects of lead poisoning has been limited by the failure to find evidence in animals of the intellectual impairment commonly found among young human victims of lead poisoning (Byers and Lord, 1943; Mellins and Jenkins, 1955). Previous experimental studies with rats have used either injections of 1.5 mg/100 g body weight of tetraethyl lead for an eight day period in adult female rats (Bullock et al. 1966) or 10 mg/100 g body weight of lead acetate for a three or four day period in weanling male rats (Brown et al. 1971). Both experiments used a water escape maze as the means of evaluating learning deficits and failed to find differences between lead injected and control animals, even though some of their animals displayed symptoms of lead poisoning.

Our notion was that the use of smaller doses of lead administered over a longer time course might better approximate the chronic lead poisoning of young humans and that the water escape maze might not prove to be the most sensitive indicator of learning deficits. Indeed, Davenport and Dorcey (1971) have reported that in experiments evaluating the effects of thiouracil injections in rats, only the Hebb-Williams maze series out of nine behavioral measures of learning used detected learning impairments in the treated animals. It was expected that in the work to be reported on here that the use of a prolonged series of small doses of lead injection and use of a Hebb-Williams maze series might better elucidate learning deficits in lead injected animals. In addition we thought it valuable to examine the effects of lead exposure at each of four developmental time periods: prenatally, during nursing, post weaning, and adulthood.

Experiment 1: Effects of lead acetate injections on learning in weanling and adult rats.

Method

Subjects: The subjects were 56 adult and 56 weanling rats obtained from the Sprague-Dawley colony in Madison, Wisconsin.

Apparatus: The apparatus used was a semi-automated version of the Hebb-Williams maze modified from the symmetrical maze designed by Davenport et al. The maze consisted of a 76 cm sq. field enclosed by wooden walls 7.5 cm high. Start/goal alleys, which were 42.5 cm long, extended from the field at diagonally opposite ends. Wooden barriers (7.5 cm high and 1.9 cm thick) of varying lengths divided the field into symmetrical maze patterns. A bolt imbedded in one edge of the barrier was inserted through the expanded aluminum flooring and fastened to hold the barrier in place. All wooden surfaces were painted flat black.

Lehigh Valley pellet feeders dispensed one 45 mg Noyes pellet per trial into a shallow aluminum dish at the far end of each start/goal alley.

A galvanized steel plate electrically isolated from the floor of the alley was placed in front of each feeding dish. This was wired to a contact detection circuit (see Giulian et al. in press). When an animal made contact with this plate pneumatic doors closed and an intertrial interval timer was started. At the end of the 10 sec ITI the doors were opened and a pellet dispensed to the opposite goal box. Observers trained in the scoring system of Davenport et al.(1970) scored the animal's errors and recorded total errors and time to the nearest sec after each trial. The animals were presented with 4 practice problems, one each day, and then each of the twelve test problems of Davenport et al. one each day. Animals were tested until they reached a criterion of 4 out of 5 errorless trials or until they ran 48 trials whichever came first.

Procedure: On arrival in the laboratory the animals were randomly assigned to one of 4 injection groups: Distilled water, 0.5, 0.8, or 1.2 mg of lead acetate per 100 g body weight intraperitoneal injections were begun on the day of entry into the laboratory and continued for twenty-one days, when testing was started, and through the subsequent 16 days of testing--a total of 37 days of injections. Each group contained 14 animals, seven males and 7 females.

During the initial 21 days in the laboratory the adult rats were slowly deprived to 85% body weight. The weanling rats were not deprived to a specific body weight percentage but were placed on a one hour per day feeding schedule starting four days before the onset of testing. Following the last day of testing each rat was placed into a metabolism cage and twenty-four hour urine samples were collected under mineral oil. All samples were immediately pipetted into glass vials and frozen, and were subsequently analyzed for the amount of delta-aminolevulinic acid (ALA) after the method of Davis (Davis et al., 1967, 1968). Urinary ALA levels have been reported to correlate well with the levels of blood lead and thus to provide an estimate of metabolically active body lead. While there has been a dispute over the precision of this test (Blanksma et al., 1970; Blanksma et al., 1970; Davis, 1970; Vincent, et al., 1970) it appeared to be an adequate gross estimate of the presence of body lead.

Results and Discussion: The animals which received the highest doses of lead acetate (1.2 mg/100 gm/day) showed signs of lead poisoning. Only 8 of 14 adults and 10 of 14 weanlings survived to complete the entire test series. Most of the animals in this group developed a hunched posture which elevated the ventral surface of the body above the cage floor. They were extremely sensitive to the handling of the abdomen and to interperitoneal injections. Those animals which died showed a severe ataxia and weight loss in the days preceding death. These symptoms were seen only among animals receiving 1.2 mg injections and not among the other injection groups. The animals which died were excluded from the analysis of the learning data.

Despite the successful production of symptoms with high doses and the use of a more sensitive learning situation--the closed-field maze series, there were no significant differences in learning. Table 1 presents the

Table 1

Measures of learning and body lead in adult and weanling rats injected with various doses of lead acetate.

Measure	Injection Condition (mg/100g)			
	0.0	0.5	0.8	1.2
		Adults		
Mean Trials to Criterion	230.9	238.1	219.0	237.1
Mean Total Errors	333.6	331.4	311,3	332.6
Mean Sec/Trial	14.3	16.6	15.6	18.7
Mean Urinary ALA (mg. %)	.32	1.30*	1.68*	1.95*
		Weanlings		
Mean Trials to Criterion	237.3	231.3	220.1	256.0
Mean Total Errors	337.9	343.2	313.1	371.8
Mean Sec/Trial	13.8	14.5	15.1	16.7
Mean Urinary ALA (mg. %)	.40	1.63*	2.40*	1.95*

* Significantly different from controls, $p < .001$, t-test.

means of all groups on trials to criterion, total number of errors, seconds per trial, and urinary ALA values. The only measure to reach significance was an increased urinary ALA level with lead injections ($F = 17.58$, $df = 3/46$, $p < .001$ adults; $F = 6.35$, $df = 3/46$, $p < .005$ weanlings).

In order to examine the possibility that there might be retention deficits although there were no differences in initial learning, 34 of the weanling rats were retested on the maze problems six weeks after the completion of the first series. The animals were distributed in conditions as follows: 10 were in the distilled water group; 9 each in the groups with 0.5 and 0.8 mg of lead acetate/ 100 g injections, and 6 with 1.2 mg/100 g injections. No animal received injections following the completion of the first maze learning series. Analyses of the savings scores indicated that there were no differences between groups in retention (t's < 1.18 for trials to criterion, df's = 14-17, p's < .20; t's < 1.53 for total errors, df's = 14-17, p's > .10). Thus neither symptomatic nor asymptomatic doses of lead administered chronically had an effect on the learning ability of adult and weanling rats, and similarly did not effect the retention ability of weanling rats when tested on the closed field maze series.

Thus the results of this study parallel those of Brown et al. (1971) and Bullock et al. (1966) that lead injected into weanling or adult rats fails to produce a learning impairment despite the fact that symptoms of poisoning can be produced. The superiority of the closed-field maze series to other measurements of learning found by Davenport and Dorcey (1971) does not serve to demonstrate a deficit here.

Since lead poisoning seems to affect younger children more than older children and adults, possibly the failure to find learning deficits with lead injections both in the present study and in the others reported (Brown et al., 1971; Bullock, et al., 1966) was because exposure to lead was administered rather late in the developmental course of the rat. In order to evaluate this hypothesis further experiments were undertaken to examine the effects of lead exposure prenatally and neonatally.

Experiment 2: Effects of prenatal lead exposure.

Method:

Subjects: The subjects were 33 female Holtzman rats which were received from the supplier on the day that a sperm plug was present.

Procedure: The pregnant females were divided into two groups: 17 received daily injections of 0.8 mg of lead acetate/100 g body weight and 16 received equal volumes of distilled water. Injections were begun on the day of arrival into the laboratory and were continued daily for 21 days. The dose of 0.8 mg/100 g was selected since it was the highest dose used previously which had produced no overt symptoms of poisoning or other visible pathologies.

Results and Discussion: None of the 17 females which were injected with 0.8 mg of lead acetate/100 g/day delivered any offspring. Thus there was no subject population on which to study potential learning disorders.

In contrast 12 of the 16 animals injected with distilled water delivered with a mean litter size of 8.6 pups. Several days after delivery was due several of the lead injected females were sacrificed and their uteri examined. In most there were signs of fetal resorbtion. The lead injected females demonstrated no overt signs of poisoning. They did not show the typical large weight gain of pregnancy, but they did gain a mean of 12.0 gm over the course of injections, a weight gain consistent with that of non-pregnant females over a similar period. Thus a dose of lead that failed to produce any signs of symptoms in adult and weanling rats previously, that had no apparent effect on the behavior of the inseminated females was sufficient to induce a 100% abortion rate.

There are reports that lead oxides were widely used in England as abortifacients in the late 1800's and early 1900's (cf. Angle and McIntire, 1964) and the one study that has compared pregnant women factory workers exposed to lead versus those not exposed to lead found a decreased success of pregnancy among the lead exposed women (Angle and McIntire, 1964). Thus the data from inseminated rats agrees with human data--that levels of lead exposure which apparently have little or no effect on the mother can have a devastating effect on the fetuses. With the failure to produce viable offspring with lead exposure during pregnancy, one is left with one developmental period to examine--the period between birth and weaning.

Experiment 3: Effects of lead acetate injections in lactating females on the learning ability of their offspring.

Method

Subjects: The subjects were 62 pups of Holtzman female rats. 26 were the offspring of 5 mothers which were injected with distilled water from the day of birth to the day of weaning, and 36 were the offspring of 7 mothers which were injected with 0.8 mg/100 g of lead acetate daily from birth to weaning.

Apparatus: The apparatus used for the closed-field maze testing of subjects in this experiment was a modification of that used in Experiment 1. A mosaic floor made of 176 electrically isolated plates was constructed with each plate running to a contact of a MAC panel. A variety of plug boards were wired to activate the plates that marked the entires to erroneous pathways for each of the twelve test problems. These in turn were wired to a contact-sensitive amplifier which recorded on a printing counter the number of errors made on each trial. A separate amplifier system using contact sensitive plates in the goal boxes recorded the running time for each trial. Thus the closed-field maze series was completely automated. No human observer was needed and thus no human bias could be introduced into the scoring as might have possible in Experiment 1. A complete description of the maze, the amplifier circuitry and validation of the automated scoring system can be found in Giulian, et al., in press. The testing procedure was identical to Experiment 1. Four practice problems were given one each day followed the twelve test problems of Davenport et al. (1970) also one each day. One 45 mg Noyes pellet served as reinforcement. The same criterion of 4 of 5 errorless trials or 48 trials was used.

Procedure: The twelve mothers who had been injected with distilled water in Experiment 2 and who had delivered successfully were divided into two groups at the birth of their litters. 7 animals were injected with 0.8 mg/100 g/day of lead acetate, while the remaining 5 were injected with an equivalent volume of distilled water. At weaning the offspring of these mothers were housed in individual cages. On day 43 they were placed on a one hour a day feeding schedule and the 4 practice problems were begun. At the end of testing these animals were placed in metabolism cages and a twenty-four hour urine sample obtained under mineral oil. This was pipetted into glass vials, frozen immediately, and subsequently analyzed for the presence of delta-aminolevulinic acid levels as before (Davis et al., 1967, 1968).

Results and Discussion: The lead injections appeared to produce no symptoms in the nursing mothers. The mean weight gain for the lead injected females was 13.3 g over the 21 days of injections, while the weight gain of the water injected females was 18.0 g. This difference in weight was not significant (U=11, n=5/7, p=.172). However, all pups of lead injected mothers showed signs of developmental retardation. They were consistently 1-2 days slower in the time of eye opening, and their mean body weight at weanling was 6.45% that of the water injected offspring. This agrees with the finding of reduced body weight and body size found in mice (Rosenblum and Johnson, 1968).

The summary of the results from the learning study is presented in Table 2. The analyses of the results indicated that rats whose mothers were injected with lead acetate during nursing made significantly more errors than did offspring of water-injected mothers (Mean = 529.2 errors-lead exposed; 450.8 controls, $t = 2.00$, $df = 60$, $p = .05$). While lead exposed animals did take more trials to reach criterion, this difference barely missed reaching significance (mean = 260.2 trials - lead exposed; 235.7 trials - controls, $t = 1.63$, $df = 60$, $.10 < p < .05$). Comparison of running times per trial indicated that there were no differences between the animals (Mean = 16.18 sec/trial - lead exposed; 17.11 sec/trial - controls, $t = .388$, $df = 60$, $p > .20$). Thus the increased number of errors made by offspring of lead injected mothers was not due to a motor deficit or a reduced level of motivation for food reward which might have been induced by lead exposure. Urinary ALA determinations made at the end of testing indicated no significant levels of ALA in the treated rats and no difference between them and control rats. Presumably the lead which had been received through maternal milk was no longer in a metabolically active form by the time of testing. These ALA results are the opposite of those found in Experiment 1. There weanling and adult rats which were injected with lead acetate showed significant amounts of ALA in their urine indicating the presence of metabolically active lead in the body. But there was no difference between lead injected and control rats with respect to learning. In the present experiment, no signs of lead metabolism are present, but the previous exposure to lead served to produce a learning impairment.

The present study did not address the question of whether and how the lead injected into the mother operated to produce a learning disorder in the

Table 2

Measures of learning and body lead in rats whose mothers were injected with distilled water or 0.8 mg lead acetate/100 gm/day.

Measures	Controls	Lead Exposed
Mean Trials to Criterion	235.7	260.2
Mean Total Errors	450.8	529.2*
Mean Sec/Trial	17.1	16.2
Mean Urinary ALA (mg. %)	.16	.18

* Significantly different from control value, $p < .05$.

offspring. However, several other studies have administered lead to nursing females and have found evidence of neuropathology in the offspring both in rats (Pentschew and Garro, 1966; Starr, et al., 1970) and in mice (Rosenblum and Johnson, 1968) and delta-aminolevulinic acid activity has been observed in suckling rats whose mothers were exposed to lead (Millar, et al., 1970). Thus it seems likely that lead is transferred by maternal milk to the nursing offspring and that lead does act to produce nervous system pathologies. However, in these studies the amount of lead to which the mothers were exposed was considerably greater than in the present study. Several (Pentschew and Garro, 1966; Starr, et al., 1970) exposed the animals to lead by adding 4% lead acetate to the maternal diet. Assuming that a nursing female ingests approximately 12.5 gm of chow per day this represents an intake of 200 mg/100 g/day. Assuming the figures available from studies of human lead ingestion (Kehoe, 1964; Thompson, 1970), approximately 4-10% of ingested lead will be absorbed into the body. This would represent an absorbtion of 8-20 mg/100 gm per day of lead. This is a range that is 10-25 times the amount that the nursing females were administered here. Thus relatively small amounts of lead that produce no symptoms in female rats, can when administered during nursing produce offspring that have retarded growth and development, and that evidence learning impairments when tested three to five weeks after weaning.

General Discussion

The work presented here shows that symptoms of lead poisoning can be produced in weanling and adult rats without producing any obvious behavioral or learning impairments. On the other hand asymptomatic doses of lead presented at earlier developmental stages have a profound effect on the offspring without producing any obvious symptomatology in the mothers. Asymptomatic doses of lead produced a 100% abortion rate in sperm positive pregnant females and produced animals with retarded development, reduced growth, and learning impairments in the offspring of treated lactating females.

The findings of learning impairments in rats exposed to lead only during nursing indicates that the rat can be considered a suitable experimental model for behavioral studies of lead poisoning if the appropriate developmental period is selected. The finding of parallels between abortion rates in lead exposed rats and lead exposed humans suggests additional evidence for parallels between the species. Since there is evidence that lead can be transferred through maternal milk (Millar, et al., 1970) and that neuropathology of the offspring appears as a result of feeding lead to pregnant females (Pentschew and Garro, 1966; Starr, et al., 1970) it seems reasonable to conclude that the learning deficits found here were due to the effects of lead administered to the mother, despite the fact that no evidence was found in the offspring of metabolically-active lead at the completion of testing which was 38 days after the termination of lead exposure.

If an analogy from rats to humans can be made, the results of this study suggest that lead exposure may be especially pernicious to pregnant or nursing humans. The exposure to lead may produce no overt symptomatology in the mother, and the offspring may not show any signs of the presence of

lead when he is evaluated for learning ability considerably later in his life. Thus some children with developmental or intellectual retardation may in fact be victims of lead exposure during prenatal or postnatal periods, yet the mother would not herself be aware of any clinical symptoms. Since no direct linkage of such retardation and the symptoms of lead poisoning may be readily observed, appropriate diagnosis and treatment of the effects of maternal exposure to lead may be difficult.

Chapter 3
A completely automated closed-field maze series for rats

A series of closed-field maze problems, developed by Hebb and Williams (1946) and later standardized by Rabinovitch and Rosvold (1951) has been used to evaluate the learning ability of rats following a wide variety of physiological and behavioral manipulations: enriched and restricted early experience (e.g., Cooper and Zubek, 1958; Forgays and Forgays, 1952) protein malnutrition (e.g., Cowley and Griesel, 1963), thyroid deficiency (Davenport, 1970), and lead poisoning (Snowdon, submitted). Davenport and Dorcey (1972) found that a series of maze problems was a more sensitive and reliable indicator of learning impairment than were other conventional behavioral tasks, such as conditioned discrimination or shuttle box avoidance.

However, despite its advantages in measuring subtle learning deficits, the Hebb-Williams maze is quite tedious to use. In the original design an observer was needed both to score errors and to transfer test animals from the goal box to the start box at the end of each trial. Davenport, Hagquist, and Rankin (1970) significantly improved this maze apparatus by the symmetrical placement of barriers within the field so that each goal box served as the start box for the next trial, thus eliminating the need to handle animals during testing. Bright illumination of the field allowed the observers to evaluate animal movements over a closed circuit television.

We found in our laboratory that observer fatigue or boredom severely limited the number of animals tested at any one time. Furthermore the reliability of data gathered under these conditions became questionable. We were led by such considerations to automate fully davenport's symmetrical maze series (Davenport, et al., 1970). The major task was to devise a versatile detection system which could monitor animal movements through a variety of maze patterns. We report here the design of a fully-automated apparatus which is simple in principle and inexpensive to construct. The total cost was below $700 including all programming and data-recording equipment. The reliability of the system is demonstrated by comparison of observer and machine scored data.

Description

Basic apparatus design

The general maze form and the methods of testing were similar to those of Davenport, et al. (1970). A 76 x 76 cm field was enclosed by wooden walls 7.5 cm in height. Start/goal alleys were placed at diagonally opposite ends of the field. Wooden barriers (7.5 cm high, 1.9 cm thick and of varying lengths) which divided the maze field into the various test patterns, were fastened firmly in place to an expanded aluminum roof by bolts embedded in one edge of each barrier. All wooden surfaces were painted flat black.

Lehigh Valley pellet feeders dispensed 45 mg Noyes pellets into a shallow aluminum dish at the far end of each start/goal alley. A galvanized steel plate (7.0 x 2.5 cm) placed in front of each feeding dish and

electrically isolated from the floor of the alley was wired to the goal detection circuit (Fig. 1b). Activation of this circuit by animal contact with the steel goal-plate marked the end of a trial--by closing pneumatic entrance gates to the start/goal alleys, by printing out data and resetting counters, and by starting an intertrial interval timer. At the end of the intertrial interval the pneumatic gates opened, a pellet was delivered to the feeding dish in the opposite goal alley, and the timing of a new trial began. Timers, stepping relays, and a Sodeco preset counter provided the simple logic to shut down equipment when a desired performance criterion was met. All programming and recording equipment was housed in a nearby room.

Construction of the maze floor was an important aspect of the automated scoring system. The floor consisted of electrically insulated pieces of expanded aluminum (6.0 cm square) which were glued with a non-conducting Neoprene adhesive to a supporting frame of Plexiglas strips. The floor was divided into 144 sections each 6.3 cm square. In most cases a single metal piece covered one section. However, to accommodate error lines that were oriented diagonally, certain floor sections were subdivided as shown in Fig. 1c. Altogether 176 individual pieces of expanded aluminum formed a mosaic over the Plexiglas frame. Adjacent metal pieces were spaced 0.6 cm apart, and the gaps were filled with Neoprene adhesive to prevent urine from electrically coupling neighboring floor plates. Wires soldered to 1/16 inch steel bolts attached to each floor plate connected that floor plate to a single contact on a MAC panel (# 109231) (Fig. 1a).

Detection circuit design

Patch boards to the MAC panel programmed the error scoring for each of Davenport's 12 test problems. A row of plates just inside an error boundary was wired to the active side (+) of a detection circuit while the row of plates just outside the boundary was wired to ground (-). When a rat stepped on both sets of plates simultaneously, the circuit was completed, registering the event on a printing counter. As an example, Fig. 1c illustrates the wiring of specific plates for test problem T-2. (Since the apparatus scored movement across error boundaries regardless of the direction taken, the entrance and departure from an alley--that is, two crossings of an error boundary--would be recorded as two distinct events.) All plates not used for defining error lines were electrically unconnected. Floor plates were wide enough to prevent a rat from jumping over them without making contact.

Identical amplifying circuits were used for both error scoring and goal alley detection. Although only two such detection circuits were included in our design, any number of additional circuits could be added to monitor specific field regions. A two-stage transistor circuit (Fig. 1b) served to detect current passing through an animal as it made contact across the ground (-) and active plates (+). (The current was minimal [< 50 μamp] and did not appear to affect the animals in any way.) When current level reached the threshold of the detector amplifier, driver transistors activated through a monostable multivibrator (one shot) operated various relay circuits in the programming and data-recording equipment

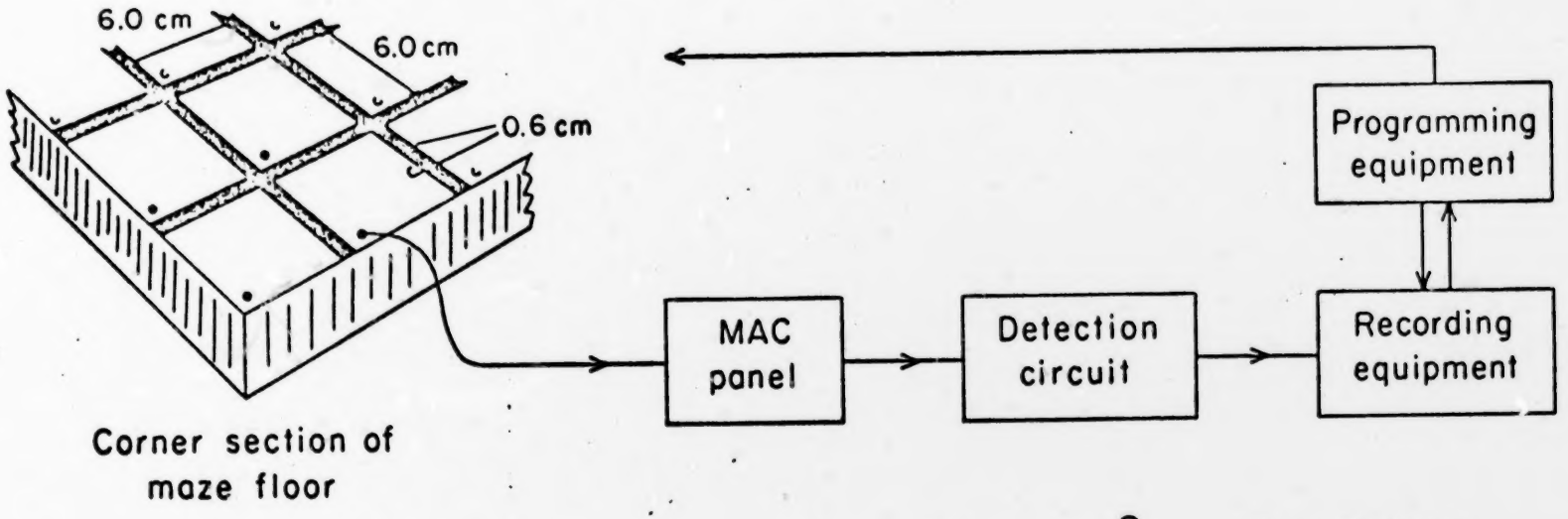
Fig. 1. A. A scheme summarizing the apparatus design. Programming equipment operates pneumatic gates and pellet feeders in the start/goal alleys.

B. Schematic diagram of the detection circuit. Specific floor plates may be "activated" by connecting them through the MAC panel to this circuit.

C. A lay-out of the maze field pattern for problem T-2. Thin lines show mosaic placement of 176 floor plates. Thick lines represent wooden barriers. Start/goal alleys are indicated outside the field in diagonally opposite corners. "Activated" floor plates labelled (+) are wired to the detection circuit; (-) plates are connected to ground. The 12 rows of (+) and (-) plates create 6 error lines for this particular pattern.

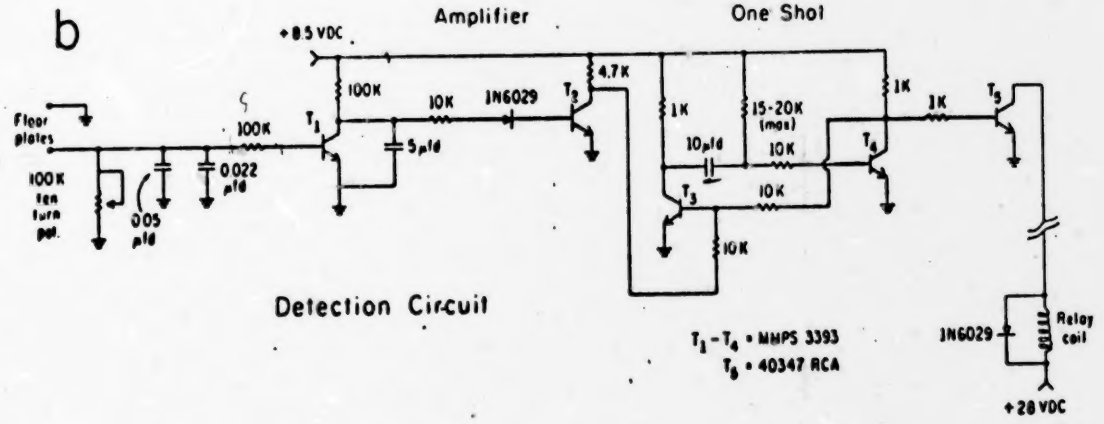
General Apparatus Design

a



Corner section of maze floor

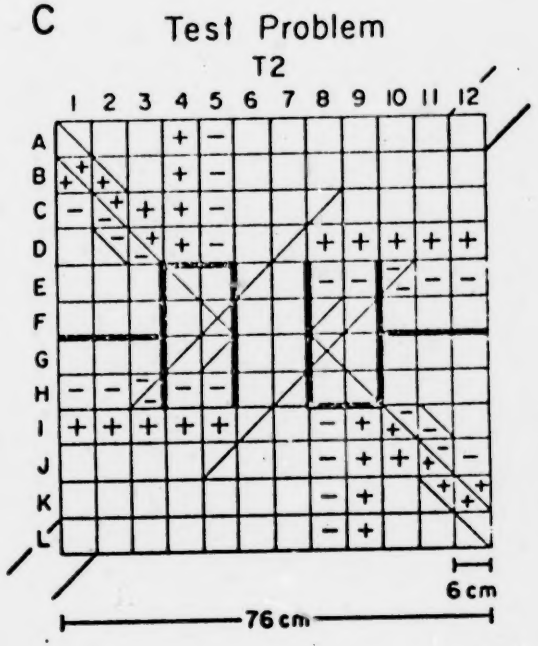
b



Detection Circuit

$T_1 - T_4$ = MMPS 3393
T_5 = 40347 RCA

C



(Fig. 1a). An RC timing circuit was included in the detection device (Fig. 1b) so that only contacts separated by at least 200 msec were scored; this prevented excessive scoring totals caused by repeated licking or pawing of the plates. The sensitivity adjustment, a 10-turn variable resistor, provided control of the detection threshold so that only paw contact (and not that from vibrissae or tail) would activate the circuit.

Several advantages of our maze design over earlier systems are worth noting. First, the complete automation of the scoring procedure reduces labor requirements and eliminates human error in data collection. Second, the patchboard programming for error boundaries is simple and easily adapted to various maze patterns. And third, the scoring system is "invisible" so that it neither distracts nor "cues" animals during training.

Supporting Data

To test the reliability of the apparatus, 50 rats, which were part of a study on the effects of lead acetate injections upon learning (Snowdon, submitted) were scored simultaneously by an observer and by the automated system for the 12 test problems. Trained observers evaluated animal performance according to the scoring procedure of Davenport, et al. (1970). After magazine training and a series of four practice problems, 23-hour food-deprived rats were presented each day with a new maze problem for 12 consecutive days. The session criterion for every problem was either 4 errorless runs out of 5 consecutive trials or a total of 48 trials.

Table 1 presents a comparison of the scoring methods. Data for each maze problem are based on the total number of trials on which at least one error was scored by either an observer or the apparatus. For example, during T-1 testing, a total of 756 trials were scored for all 50 rats, with the machine recording 1937 errors and the observer reporting 1299 errors. The product moment correlation (r) for T-1 between machine-scored and observer-scored data on a trial-to-trial basis was .811. Such correlations were statistically significant ($p < .001$) for all maze problems. We attribute this difference to the fact that the automated apparatus detected both entries into and departures from an alley while the observer was instructed to score only entries (Davenport, et al., 1970). Moreover, our detection system was sensitive to paw contacts that could not easily be scored by an observer looking down upon the maze field. Despite the variations in the absolute number of errors scored, the two methods are highly correlated and adequately reflect performance on a trial-to-trial basis. Even higher correlations appear when subject by subject correlations are examined. Thus regardless of the differences in scoring, the automated maze detects the same deficits.

Two problems have appeared with use of the maze. First, a small proportion of the animals tested (<2%) failed to trigger the error plates even when the sensitivity was increased. Washing their feet with a damp paper towel prior to testing eliminated this problem. Secondly, under conditions of excessive humidity (>90%) the condensation of moisture caused some shorting between plates, making operation impossible until the humidity was decreased.

Table 1

Trial by trial and subject by subject correlations of the automated scoring versus the observer scoring procedure.

Test Problem	N Trials	Total Machine scored errors	Total Observer scored errors	r * (Trial by trial)	Total Subjects	r * Subject by subject
T-1	756	1937	1299	.811	50	.985
T-2	988	2033	1288	.850	50	.924
T-3	875	1771	1018	.811	50	.903
T-4	773	1491	1065	.863	50	.956
T-5	464	603	404	.835	50	.976
T-6	992	2480	1472	.772	50	.954
T-7	759	1435	1156	.835	50	.908
T-8	811	1196	917	.769	50	.836
T-9	657	1003	894	.812	50	.891
T-10	782	1481	1114	.869	50	.957
T-11	607	748	554	.868	50	.932
T-12	912	1385	1215	.757	50	.925

* r = product-moment correlation. All correlation coefficient values show $p < .001$.

In subsequent experiments, with more than 300 animals, the maze has successfully shown performance differences in rats with neonatal cortical lesions (D. Giulian, unpublish-d observations), in siblings from mothers injected with lead acetate (Snowdon, submitted), and in rats suffering from protein deficiencies (S. R. Gilman and C. T. Snowdon, unpublished observations). In addition, the maze can be readily adapted for the testing of other rodents or for the monitoring of open-field activity. Our improvements in the design of the serial maze provide a convenient, sensitive, and inexpensive apparatus which allows the testing of large numbers of animals.

Chapter 4
Lead Pica Produced in Rats

Lead poisoning resulting from pica has been estimated to affect as many as 225,000 young children each year (Oberle, 1969) leading to severe and irreversible retardation (Mellins and Jenkins, 1955; Byers and Lord, 1943; Berg and Zappella, 1964) and clear evidence of brain damage. Why children should voluntarily ingest lead is not clearly understood (Pentschew, 1965; Perlstein and Attala, 1967; Thurston et al. 1955). The most common explanation is that the failure of the mother to provide proper supervision of the child or the proper emotional support of the child (Millican, et al. 1956) coupled with a young child's predilection for mouthing and ingesting non-food substances (Lourie, et al. 1963, Neumann, 1970) leads to the maladapted behavior.

An alternative to the inadequate maternal care notion is that the pica might have a nutritional basis. Animal picas have frequently been determined to have a nutritional basis (Green, 1925). There are two leading nutritional candidates for lead pica. Iron deficiency anemia is commonly found in conjunction with lead poisoning and was suggested as a possible predisposing factor to lead pica (Watson, et al. 1958). However, Gutelius, et al. (1962 a) failed to find evidence of iron deficiency in the diets of children with pica and failed to demonstrate a subsequent reduction of pica with iron injections (Gutelius, et al. 1962 b).

The other nutritional candidate is calcium. There have been several indications of a metabolic interaction between lead and calcium. The addition of calcium lactate to the diet of lead injected rats reduced their body lead levels (Lederer and Bing, 1949). Rats placed on a low calcium diet showed an increased toxicity to lead exposure manifested by increased body lead in blood, soft tissues, and bone, and increased urinary levels of delta-aminolevulinic acid. Kidney damage was also found, yet the lead levels used did not produce damage in rats with normal dietary calcium levels (Six and Goyer, 1970).

The present experiments examine the possible nutritional basis of lead pica and the specific role of calcium. Three questions were asked: (1) Do normal weanling rats demonstrate any preferences for lead solutions? (2) Does calcium deficiency or iron deficiency produce an increased lead ingestion? (3) Do animals detect the presence of lead in their bodies and regulate their lead levels through their ingestive behavior either by reducing voluntary lead ingestion or increasing calcium ingestion?

97 Sprague-Dawley male and female weanling rats were studied in a two-bottle preference situation. Five solutions were presented with distilled water as the alternative in a Latin Square design that was repeated so that solutions appeared once on each side to control for possible side preferences. The test solutions were distilled water, .08%, .16%, .32%, and .64% wt/vol lead acetate solutions. Since lead acetate began to precipitate as insoluble lead hydroxide at the highest concentrations, 3 ml/l of 5% acetic acid was added as a buffer to all solutions, including distilled water.

All animals were received at weaning and adapted for 25 days before the start of any testing. During this time mineral deficiency or injection manipulations were begun. All manipulations continued through the test period. The deficient diets used were General Biochemicals Low Calcium Test Diet (.002% calcium) and Low Iron Test Diet; the normal diet was Wayne Lab Blox (1.2% calcium). The protein, fat, and carbohydrate content was similar in all diets. All diets were available ad lib. Twenty-four hour fluid intake data were converted for analysis in three ways: (1) the mg. of lead acetate ingested per 100 g. body weight per day; (2) the amount of test solution ingested as a percentage of total fluid intake; and (3) the total fluid ingested per 100 g. body weight per day.

Thirty six non-deficient weanling rats were presented with the test solution series. They decreased their percentage intake of lead solutions with increased concentration (Table 1, top line), but the compensation was not precise. Thus, there was a significant increase in the mg/100 g. of lead ingested with increased concentration ($F = 10.46$, $df = 3/177$, $p < .001$). This finding of increased lead ingestion with increased concentration was found with every group of rats tested regardless of manipulation. The total daily fluid intake remained constant over all solutions. Non-deficient rats do not show a preference for lead solutions and reduce the percentage intake to 11-12% with high concentrations.

The second series of experiments examined the nutritional deficiency hypothesis. 27 calcium deficient and 16 iron deficient rats were used. Intake data of these animals were analyzed with the non-deficient data presented above. The results are presented in the second section of Table 1. There were no differences between the iron deficient and control weanlings in mg. per 100 g. per day of lead ingested, in percentage of test solution ingested, or in total fluid ingestion. Body weights of the iron-deficient animals were significantly lower than non-deficient rats ($t = 6.968$, $df = 50$, $p < .001$).

However, the calcium deficient weanlings showed a significant increase in the amount of lead ingested per 100 g. per day ($F = 10.43$, $df = 1/61$, $p < .005$). They also ingested proportionally more lead at higher concentrations than did control animals ($F = 4.096$, $df = 3/177$, $p < .001$). Calcium deficient rats ingested a larger percentage of their fluid intake from lead solutions than did control animals ($F = 36.30$, $df = 1/61$, $p < .001$) and showed a disproportionate increase at higher concentrations ($F = 10.13$, $df = 3/177$, $p < .001$). t-tests performed at each concentration between calcium deficient and control animals showed a significant difference in each measure at every concentration (t's > 4.21, $df = 61$, p's $< .001$). Two subsequent replications with minor changes in procedure have produced the same pattern of results.

Calcium deficiency imposed on weanling rats reliably produced a significant increase in the amount of lead per 100 g ingested at all concentrations. The calcium deficient weanlings ingested an average of 33.5 mg lead acetate per 100 g. per day. If the values found from humans on absorbtion of lead from the intestines (4-10%) are used (Kehoe, 1964; Thompson, 1971), the rats absorbed 1.34 to 3.35 mg of lead per 100 g per day. This level is greater than the 1.2 mg per 100 g. per day injections used by Snowdon (submitted) which produced severe symptomatology and death in many animals.

Table 1

Group	mg. lead acetate/100 gm/day Solution				Test solution ingestion as percent of total (ml total fluid ingestion/100 gm/day) Solution					Body weight gms
	.08%	.16%	.32%	.64%	HOH	.08%	.16%	.32%	.64%	
Controls	4.00	6.20	9.60	12.60	53.9 (17.8)	26.1 (18.5)	22.3 (17.4)	12.9 (18.1)	11.2 (17.4)	211.1
Deficiency Experiments										
Calcium deficient (N=27)	7.15***	8.81***	12.52***	33.53***	52.1 (16.9)	57.4*** (15.4)	44.6*** (13.9)	33.0*** (12.5)***	35.6*** (15.3)	122.8***
Iron Deficient (N=16)	2.98	5.32	5.10	13.49	60.3 (17.4)	21.2 (17.4)	19.4 (17.4)	10.1 (16.0)	12.9 (15.8)	145.6***
Injection Experiments										
HOH Injections (N=9)	3.28	7.57	9.30	9.85	59.0 (22.2)	20.3 (20.9)	25.0 (20.1)	15.1 (19.7)	8.6 (18.5)	195.2
Lead Injections (N=9)	5.63	9.27	13.86	18.49	50.4 (21.0)	31.4 (22.6)	25.6 (23.9)	19.9 (21.8)	14.3 (22.0)	171.8

*** Significantly different from control values by t-test, all p's < .001.

Thus the calcium deficient rats in the present study voluntarily ingested lead at toxic levels. The calcium deficient rats had lower body weights than either the control rats (t = 11.586, df = 61, p < .001) or the iron deficient rats (t = 3.792, df = 41, p < .001).

The final series of experiments examined the effects of lead injections on the ingestive behavior of weanling rats. If animals can regulate the level of lead in their bodies through their ingestive behavior, then imposing an increased body lead level on animals should produce a reduction of voluntary lead ingestion below the already low levels of normal animals. If calcium and lead are interrelated, then an animal with an increased body lead load might compensate with an increased voluntary calcium ingestion.

Eighteen weanlings were divided into a lead injected and water injected group with the lead injected animals receiving .8 mg/100g of lead acetate daily for 25 days prior to the start of testing. Water injected rats received equal volumes of distilled water. No significant differences were found between the groups on any of the measures of ingestion (Table 1, 3rd section). From these data it would appear that lead injected rats cannot compensate for an increased lead load by reducing their voluntary lead ingestion.

Twenty weanlings were divided into lead injected and water injected groups. Following 25 days of injection they were tested in the two bottle situation with distilled water, .6%, 1.2%, and 2.4% solutions of calcium lactate in a repeated Latin Squares design. Analysis of the data indicated an increased calcium ingestion in lead injected rats (F = 5.28, df = 1/16, p < .05), an increased calcium ingestion by female rats (F = 6.52, df = 1/16, p < .025) and an increased calcium ingestion by lead injected female rats (F = 6.09, df = 1/16, p < .05). No sex effects were found in the previous results. t-tests between injected and non-injected females indicated increased intake in mg of calcium lactate per 100 g. per day for the .6% (lead injected mean = 83.9 mg; HOH injected mean = 38.0 mg, t = 2.500, df = 8, p < .05) and the 1.2% solutions (lead injected mean = 232.0 mg, HOH injected mean = 100.4 mg, t = 5.354, df = 8, p < .001) with the intake at 2.4% barely missing significance (lead injected mean = 384.4 mg, HOH injected mean = 208.0 mg, t = 2.081, df = 8, .10 > p > .05).

While the appearance of a sex difference is puzzling, the increased calcium intake of lead injected rats corresponds to reports of many children with lead pica drinking large amounts of milk (Mellins and Jrnmins, 1955; Gutelius, et al., 1970). Indeed it has been suggested that such milk ingestion might be the cause of the iron deficiency anemia found with lead pica (Werkman, et al., 1964).

These experiments demonstrate that weanling rats do not normally ingest lead when an alternative, non-lead solution is available, nor do they compensate for increased body lead loads by reducing still further their voluntary lead intake. The low level of lead ingestion by normal rats probably does not represent an active regulation of lead levels, but rather taste qualities of even low concentrations of lead are probably aversive. Osmotic factors were not responsible for the low lead intakes since all solutions were in the hypotonic range.

Only a calcium deficiency imposed on weanling rats produced a markedly elevated voluntary lead ingestion. Deficiency, per se, did not lead to the increased ingestion since iron deficient weanlings did not differ from controls. This suggests an involvement of calcium in the production of lead pica. This is further supported by the finding that at least female weanlings will increase voluntary calcium consumption when injected with lead.

Aside from the finding of increased milk consumption in some children with lead pica, there is no direct evidence of calcium deficiency leading to lead pica in humans. However, lead pica is most common among low-income children and a survey of studies of mineral nutrition in the United States (Davis, et al., 1969) indicated that almost 60% of the low-income households surveyed provided less than the minimum recommended level of calcium, a degree of failure greater than any other nutrient surveyed. Thus low income families are likely to provide inadequate calcium levels for their children.

Recent research in specific hungers in animals suggests a mechanism whereby a lead pica might be maintained in the absence of an obvious, symptomatic calcium deficiency. A nutrient deficiency induces the organism to seek other food substances, sampling until one source relieves the aversive symptoms produced by the deficiency. Such an animal will continue to ingest this symptom relieving food unless and until it also produces negative symptoms through nutritional inadequacies of its own (Rozin and Kalat, 1971). Such an interpretation of lead picas would argue that a child deficient in calcium may discover that lead relieves some of the symptoms of calcium deficiency. Thus, lead becomes a highly rewarding substance which the child continues to ingest beyond the duration of the calcium deficiency. But lead brings negative symptoms of its own. Why then should pica continue to the point of severe poisoning? Another experiment suggests a possible solution. Calcium deficient rats were allowed to drink calcium solutions to restore their deficiency. Injections of lithium chloride were then administered concomitantly with calcium ingestion, a procedure that normally induces a long lasting aversion for the solution it is paired with. However, in the case of calcium deficient rats drinking calcium, the aversive effects of lithium chloride did not reduce calcium intake (Frumkin, 1972). Possibly for the child with lead pica the reinforcing effects of lead ingestion relieving calcium deficiency are sufficiently powerful that the negative effects of continued ingestion cannot overcome the impetus to ingest lead.

Chapter 5: Further Studies of Lead Pica

The preceding chapter presented our results on weanling rats, indicating that calcium deficiency was sufficient to induce a significant increase in voluntary lead ingestion. It also presented a model by which a child might acquire a lead ingestion habit. Namely, the child is subjected to a calcium deficiency and discovers through its own behavior that some substances, which happen to contain lead, remove the symptoms of calcium deficiency. In parallel with results of animal studies this relieving of symptoms by pica becomes sufficiently reinforcing that the subsequent appearance of symptoms due to lead ingestion does not inhibit the pica. Frumkin (1972) showed that calcium deficient rats who had learned to ingest calcium could not be induced to stop drinking calcium when its ingestion was paired routinely with nausea producing drugs.

The present chapter extends our initial results by studying whether picas could be produced in adult animals and by attempting to see if the learning model presented above does hold with respect to lead. In both cases the results to date have been negative.

Experiment 1

Method and Results:

80 adult male and female rats of the Sprague Dawley strain were tested under the two bottle choice tests described above for weanling rats. Twenty rats were tested at one time in a repeated 5x5 Latin Square design. The first group of rats was simply tested for their preference of leaded solutions versus distilled water and they displayed the same function as the weanling rats. That is there was a decrease in lead intake both as measured by the number of gm of lead per 100 gm body weight ingested and in terms of the percentage of leaded solutions ingested as concentration was increased. Both age groups of rats then seem not to prefer leaded solutions to distilled water.

A second group of adult rats was tested with the solutions available for only one hour each day. The logic behind this manipulation is that it was possible that with a solution available for twenty-four hours some post-ingestive consequence of the lead ingestion might modulate subsequent ingestion. Thus lead might taste good to the animal but by virtue of postingestive symptoms the animal instead of showing a preference would display an aversion. The one hour test was thought to minimize the possible post-ingestive effects of lead and to show the operation of taste factors alone The results indicated that while total fluid ingestion was decreased as a function of making fluids available for only one hour each day, the relationships between concentration and percentage of each solution ingested remained the same as with twenty-four hour ingestion. Thus percentage of lead ingested decreased as the concentration of lead increased. This indicates that the aversion rats showed to lead solutions must be primarily due to negative taste factors.

In the next experiment adult rats were injected with .8 mg/100 gm of lead acetate for twenty-one days and then tested for their response to lead solutions. As with the weanling animals there was not the expected decrease of lead ingestion that would be predicted if the rats were able to sense their body lead levels and change their ingestive behavior accordingly. Rather levels of lead ingestion remained comparable to those of non-injected animals.

In the final set of experiments adult rats were deprived of either calcium or iron and then tested. In contrast to the weanlings, there was no increased ingestion by the adult calcium deprived animals. The percentage and total lead intake remained the same as those of non-nutrient deprived control animals. Thus the effect of calcium deprivation on increased lead ingestion appears to be present only in young rats.

Experiment 2

The next series of experiments examined the effects of imposing calcium deficiencies for longer periods of time - through pregnancy, lactation, and weaning and on the effects of calcium restoration on lead ingestion. It might be expected that if the lead pica is a learned behavioral response to a calcium deficiency that the behavior would then continue beyond the immediate period of deficiency.

In two separate replications pregnant females were placed on a low calcium diet. They remained on this diet through pregnancy and lactation and the offspring were continued on the low calcium diet until 42 days of age. At this point preference testing began. In both replications the same finding as before was obtained - that calcium deficient rats ingested large quantities of lead relative to the offspring of non-deprived control mothers. When these calcium deficient rats were returned to a normal diet (Purina Lab Chow) and subsequently tested for preferences, there was no indication of increased lead ingestion. Thus there was no carry-over of the posited learned lead ingestion beyond the period of calcium deprivation. Two possibilities are suggested: either the hypothesis about the acquisition and maintenance of pica is incorrect and needs to be discarded, or the very high levels of calcium that appear in commercial diets for laboratory rats are not related to the natural history of children with pica. More likely if they have been deprived of calcium in adequate amounts at one point in their life it is unlikely that they would be provided with an over-compensation later on The appropriate analigue for rats would be to maintain them, not at deficient levels of calcium nor at extremely high levels, but to have them receive calcium levels just below the minimum recommended daily intake. If in these animals a pica persisted even after the restoration of some calcium, it would account for the fact that many children with pica do not show symptoms of calcium deficiency yet persist in pica. A study on this point was not possible within the time limits of the grant.

Experiment 3

This third series of experiments attempted to determine whether there was any basis for the model of learned lead intake proposed earlier. The basic paradigm used was modelled after that of Frumkin (1972). Rats would be made deficient in calcium, then provided an education period with one of three solutions 2.4% calcium lactate, .32% lead acetate or distilled water over a seven day period to "educate" the animal about the effects of that solution and then a preference test where some conditioning manipulation might be made.

In the first experiment of this series twenty-four rats were placed on a calcium deficient diet for 25 days post-weaning. At the end of this period, while still on deficient diet they were offered either lead acetate, calcium lactate, or distilled water as an exclusive solution for seven days and were then tested for one hour a day for their preferences for the solution they were "educated" with versus distilled water. Those rats which had been calcium deficient but had been allowed to drink calcium lactate solutions showed a clear preference for calcium over water. This result was expected given previous work on calcium deprivation. However, the animals which had been exposed to lead solutions only failed to show a preference for lead when offered a choice between lead and water. Since the conditioning phase of this experiment was predicated upon the existence of a preference for lead which did not appear, the experiment was terminated.

In the second experiment weanling rats were placed on a calcium deficient diet and offered .32% lead acetate, 2.4% calcium lactate, or distilled water as the sole fluid available for a period of twenty-five days. During this period it was predicted that if lead were really benefitting the calcium deficient animals that their weight gain and growth should be greater than those animals on a calcium deficient diet having only distilled water to ingest. In contrast to this prediction, however, the lead ingesting rats gained weight at the slowest rate of the three groups being significantly behind the weight gain of the water ingesting rats. Two conclusions are possible - one is that in the context of this experiment lead ingestion does not serve any therapeutic function. The second is that the lead which was shown in Chapter 4 to have been aversive in taste to the rats, when offered as the only solution for ingestion prevented the rats from ingesting normal fluid intakes. The failure to ingest adequate fluids inhibits the amount of food eaten and since reduced intakes were found with this group the failure to gain weight may have been due to the effects of the taste of the particular lead solution used. Subsequent tests will have to find a lead solution or an adulterated lead solution that is initially palatable to rats. Thus the question of whether lead is actually beneficial to calcium deficient rats has not yet been determined.

The third series of experiments attempted to get at the question of the condition-ability of the pica. The design was to find solutions of lead and calcium that would seem to be equivalent in taste to normal rats, and then deprive weanling rats of calcium, educating them with one or the other solution for several days and then begin a three day testing cycle with the

training solution presented on one day, distilled water on another day, and a choice between solutions on the third day. Following one of the drinking days, one half of each group would receive lithium chloride injections that induce nausea, and following the other drinking condition they would receive sodium chloride control injections, with the third day of choice indicating the degree to which conditioning occurred. This sequence would be repeated up to ten times. One half of the animals in each group would get lithium chloride paired with water, the other half would have it paired with the test solution either calcium or lead. Again in practice we were not able to equate adequately the taste values of calcium and lead. Nor were we able to establish Frumkin's result of the calcium drinking rats showing no response to lithium chloride injections following calcium ingestion. Thus we could not extend our model without being able to produce results obtained by Frumkin. Finally, in every case regardless of injection condition, the lead ingesting rats always drank water in preference to lead solutions. Thus we have been unable to establish our conditioning model as a plausible interpretation of the increased lead ingestion seen with calcium deficiency.

Despite the uniform failure of the experiments in this chapter, they do not yet rule out the validity of our interpretation of lead pica. In the original study (Chapter 4) lead ingestion increased with calcium deficiency, but never reached the point of lead solutions being preferred to water. Taste factors were shown to be aversive to the animals. And animals were exposed to the strongest solutions for only two twenty-four hour periods each. It is clear from the second set of experiments reported here that the phenomenon of increased lead ingestion with calcium deprivation is still obtainable, though the presence of high levels of calcium in the diet or provided in water immediately eliminates lead ingestion. We need to perform additional experiments where the negative taste of lead acetate is masked with a stronger flavoring such as vanilla, or when a sweetener such as saccharin is provided, and to test the preference for other forms of lead compounds. Once a good tasting form of lead is found or created by adulteration; most of these experiments will need to be repeated. Does lead continue to be palatable or preferred by calcium deficient rats when presented over a long period of time? Will lead solutions serve to prompt growth and weight gain of calcium deficient rats similar to those rats that have calcium available? Can a preference for a tasty form of lead be conditioned against with pairings of lithium chloride injections in calcium deficient rats. In this latter case if adulterated forms of lead solutions are needed to make them palatable to rats, then similar adulterations will be necessary for the distilled water control solutions. And it will be necessary to repeat the experiments where calcium deficient rats were given calcium replacement therapy using sub-minimal doses of calcium replacement and more palatable forms of lead solutions to determine whether the increased calcium ingestion can appear beyond the termination of complete calcium deficiency. The finding of positive results to each of these questions would represent the confirmation of our model of lead pica.

Chapter 6: Effects of Degree of Protein Malmutrition and Age of Administration of Learning in Rats.

The preceding chapters have been concerned with the effects of lead both on learning abilities in rats and on the possible nutritional basis for voluntary lead ingestion. The concern with nutritional variables and the discovery that lead exposure was most effective at a particular developmental stage lead us to a consideration of the effects of protein malnutrition on learning in rats.

There is adequate evidence that protein deprivation curtails brain cell development during the critical period of brain growth in rats - until the age of weaning (Winick, 1969). But the leap from physiological changes to behavior is often difficult and complex. The literature on the behavioral effects of protein malnutrition is complex and confounded by the use of various types of diets, various tests to evaluate behavioral deficits, various developmental periods during which protein malnutrition is provided, and various species being tested. As a result definitive statements about the effects of protein malnutrition and learning have been difficult to make. Thus, Collier and Squib (1968) fed chickens a standard poultry diet from hatching and another group a protein deficient diet of ground corn and corn oil. After two weeks 25% of the experimental group had died, and the rest were rehabilitated on the normal diet. When the chickens were tested on a visual discrimination task there were no differences between malnorished and control animals in learning or performance measures. Barnes et al. (1968) fed pigs diets low in protein, low in protein and calories, a normal diet, or restricted amounts of normal diet from the third to eleventh week of life. Aniamls were then rehabilitated on a normal diet. No differences were found between groups in the acquisition of a conditioned response, though the normally fed pigs showed a faster extinction rate than the protein deprived pigs. However on a conditioned avoidance and bar press tasks, the amlnourished pigs did show a slower acquisition.

Cowley and Griesel (1968) found that female rats maintained from day 21 through the weaning of their first litters (on 13% protein versus 20% for controls) produced offspring that made significantly more errors, but were also significantly slower on the Hebb-Williams maze series. Caldwell and Churchill (1967) fed pregnant rats either a low protein diet (14.4%) or a high protein diet (47.9%) until partuition when the rats were placed on standard lab chow. The offspring were tested on a Lashley III water maze and the protein deficient rats did less well, though the difference was qualitative rather than quantitative. Barnes et al. (1967) fed pregnant rats a diet of 25% protein through the end of nursing. Some had litters of eight; others of six. At weaning the pups were divided into a low protein group (3-4%) and a normal protein (25%) group. After eight weeks dietary rehabilitation was instituted and a Y water maze was used. In a position reversal task all post weaning protein deficient males made more errors than controls and protein deprived females from large litters were poorer than others. In a visual discrimination task, protein deprived males, but not females, showed poorer performance.

In our study we selected the Hebb-Williams maze with a food reward which has been shown by Davenport and Dorcey (1971) to be the most effective diagnostic of learning deficits in manipulated rats. We used the automated version of this maze described in Chapter 3. In addition two levels of protein malnutrition were imposed. Since previous work defined low levels of protein as anywhere between 3% and 14% we used two levels of malnutrition--3% and 14%. Finally to determine the effects of malnutrition applied at each developmental stage we manipulated the protein levels during pregnancy, during nursing, or following weaning. The results reported below are from an initial study. Additional data are still being gathered on a larger sample of rats.

Method

Subjects: The subjects used were the offspring of pregnant Holtzman rats. The pregnant females were brought to the laboratory on day one after sperm were determined present in the vagina. In the initial study there were a total of 65 offspring of 20 different sperm positive female rats used.

Procedure: A given female was assigned to one of three dietary conditions to be fed during pregnancy and was assigned to one of the three dietary conditions during nursing. Additional females were fed on laboratory chow during both pregnancy and weaning and their offspring assigned to one of the three dietary conditions following weaning. Three diets were used: Purina laboratory chow in powdered form which produces 2.9 cal/gm and contains 24.5% protein; General Biochemicals Low Protein Test Diet which produces 3.9 cal/gm and contains 3.5% protein (the low condition); and an equal weight mixture of these two which produced 3.4 cal/gm and contianed 14.0% protein (the medium diet). All animals had access to these diets at all times. Offspring of mothers who were given low or medium test diets during pregnancy or nursing were returned to normal Purina Chow at weaning and kept on that diet until the time of testing.

The testing began 21 days after weaning (Day 42). Each rat was placed on a one hour 1 day feeding schedule and given magazine training in the goal box of the automated Hebb-Williams maze (Giulian et al. (in press)) and then on subsequent days was run twelve trials on each of four practice problems. For the next twelve days each rat received training on a different problem, one each day. Rats were run to a criterion of four out of five errorless runs or a total of 48 trials whichever came first. Printing counters automatically recorded the time to run on each trial and the number of errors made on each trial.

Data were analyzed by Analysis of Variance and subsequent t-tests for measures of body weight, total trials to criterion, total numbers of errors and the mean running speed per trial.

Results

The results presented here are from the preliminary study in which used a total of 63 subjects. Work still in progress is increasing the sample size

so that more accurate statements about age of treatment, degree of malnutrition and possible sex differences can be made. While there was only a small amount of difficulty in breeding success of pregnant females receiving low levels of protein, we found that every attempt to feed a nursing female on low protein diet resulted in failure, due to the mother's cannibalization of her young - possibly using them as a source of protein. Thus only two dietary conditions were possible in the preweaning period.

There was a significant effect of prenatal diet on the body weights of the animals at the time of testing (Day 42) with low and medium protein animals weighing significantly less than normal protein groups (82.4 gm for Low; 90.7 for Medium, 127.4 gm for Normal). No significant weight difference appeared as a result of the type of preweaning (nursing) diet available. And no differences appeared in body weights as a function of post weaning dietary manipulation.

In the total trials to criterion measure the only significant results that appeared was in the prenatal manipulation ($p < .005$). Neither postnatal or postweaning manipulations had any effect on trials to criterion data. The means for the prenatal groups were: Low = 352.2 trials to criterion; Medium = 3.5.3 trials; and Normal = 290.4 trials. In the analysis of total errors four significant effects appeared. Prenatal diets were significant ($p < .005$ with means: Low = 1110.0 errors; Medium = 725.7 errors; Normal = 765.7 errors. The low protein scores were significantly higher than those of the other two conditions. The postnatal diet also was produced a significant difference in errors ($p < .005$) with means: Medium = 876.5; Normal = 635.7 errors. In addition there was a significant sex effect ($p < .001$) with males making fewer errors than females (698.3 versus 883.1). And there was a significant prenatal diet by postnatal diet interaction ($p < .025$) with the animal receiving the lowest total protein across both conditions making the most errors. There were no significant effects affecting postweaning manipulations.

Quite important in dealing with malmourished animals in an appetitive situation is the necessity to demonstrate that they were under no motivational impairment. Hence, we used running speed as a measure of motivation. If running speed is no different in low protein groups or is significantly faster than that of normal protein groups, then a deficit in food motivation cannot be invoked to account for the decreased maze performance. Analyses of the time per trial data indicated several significant effects. With prenatal dietary manipulations the normal protein rats took significantly longer to run each trial than did low or medium protein animals (Normals = 22.9 sec/trial; Medium = 14.9 sec; Low = 17.3 sec). With postnatal manipulations the medium protein group took significantly longer than the normal group (21.6 sec versus 13.0 sec/trial). With postweaning manipulations the low and normal protein groups were significantly faster than the medium group (Low = 15.0 sec/trial, Medium = 29.1 sec and Normal = 16.8 sec). Thus across conditions there is no consistent effect of protein deficiency on running spbed and in each case the low protein group was significantly faster than or equal to the normal group, yet in the prenatal manipulation the low protein animals made significantly more errors and took more trials to reach criterion.

Discussion

The results from this preliminary study indicate that low levels of protein administered during nursing do not allow the development of viable litters, and both prenatal and postnatal protein malnutrition have a significant effect on body weight at Day 42. Prenatal malnutrition produced learning impairments both based on total trials to criterion and total errors, while postnatal protein malnutrition produced a significant increase in error scores only. No effects of protein malnutrition on learning were found when the malnutrition was imposed past weaning. The learning impairments could not be accounted for by a motivational impairment since the low and medium protein prenatal groups both showed significantly faster maze running times than the normal protein prenatal group.

These results indicate that the most severe effects of protein malnutrition on learning ability result when protein malnutrition is imposed prenatally. Even after a period of rehabilitation on a normal diet, prenatally protein malnourished rats showed a reduced body weight. The manipulation during the postnatal period is not as clear cut, due to the inability to raise low protein animals. It is not clear whether the effects of protein malnutrition are as great during this period as they were during prenatal periods. It was clear though that the degree of protein deprivation affected the severity of deficit displayed. Since previous studies have defined low protein conditions as ranging from 3%-14% protein and since we found here such clear differences between these two levels of protein, it is important to carefully consider the degree of protein deficiency being imposed when interpreting results. One person's low protein condition may in fact mean a rather minimal change in diet while another's might indicate a very severe effect. In general from our results and those available from other sources it would seem that the earlier and more severe the protein deprivation imposed the greater the degree of learning deficit displayed. In the present case the use of an appetitive task which had been previously shown to be the most sensitive indicator of learning deficits with other types of manipulations, proved to be appropriate. Clear differences were found and could not be explained away in terms of a motivational impairment since running speeds of the most severely deprived groups were equivalent to or faster than those of non-deficient rats.

With respect to implications for humans, both this and the learning research with lead described in Chapter 2 indicate that the time after weaning is not as sensitive to manipulations of the nutritive state as are prenatal and postnatal periods. With lead there was a 100% abortion rate occurring with non-symptomatic levels of injection into pregnant females. With protein malnutrition there was clear learning deficit measured both by trials to criterion and by total errors. In offspring whose mothers were injected with lead during nursing there were learning deficits in terms of the total number of errors, and this same result appeared with the postnatally protein deprived groups. In both the lead and the protein malnutrition studies there were no effects when the manipulations were made after weaning. This is not meant to imply that both lead and protein deprivation act by similar means to produce deficits in learning, but rather to point out the susceptibility of rats and possibly humans to these environmental and nutritional manipulations seems to be the greatest at the earliest developmental ages. For humans these results would indicate the need for special diagnostic care

from the very onset of pregnancy to insure that protein nutrition was adequate and that exposure to environmentally virulent compounds such as lead was kept minimal. These earliest periods may be those when the child is most affected.

Chapter 7
Conclusions

The results of the work described in this report can be briefly summarized:

1. Lead acetate injections can produce symptoms of lead poisoning with the highest dose levels in adult and weanling rats, but despite this fail to produce any learning deficits when measured by a Hebb-Williams maze. This is in accord with the two published reports on the effects of lead on learning ability. However, when sub-symptomatic dose levels were injected into pregnant female rats all mothers aborted. And when these same low levels were injected into nursing mothers, they produced offspring which weighed less at weaning than control injected offspring and they demonstrated a deficit in Hebb-Williams maze performance. Thus lead exposure does not seem to affect animals past weaning, but to be devastating at earlier developmental stages, even when there is no apparent effect on the mothers receiving the injections. The results of this work as described in Chapter 2 are in press with Pharmacology, Biochemistry and Behavior.

2. A major difficulty in doing research on learning impairments using animal models of human problems is the development of adequate assessment techniques. Davenport and Dorcey (1972) have shown that the Hebb-Williams maze series is the most sensitive detector of learning deficits they have found. Yet this method is quite cumbersome in practical use. Because a different problem must be used each day, it has been necessary to have on hand human scorers to follow the subjects on each trial and record errors. As we began the work above on learning in lead poisoned animals it became readily apparent that our scorers fatigued readily and often were inaccurate. The number of animals that could be tested at any one time was limited by observer fatigue. We were thus led to the development of the first completely automated Hebb-Williams maze using a mosaic of floorplates which are wired to a MAC panel which can then be programmed for any pattern of errors desired. In comparing this automated system which amplifies a very small current that passes through an animal when it touches the floor plates on either side of an error line, with hand scored data we find very high correlations on both a trial by trial basis and subject by subject basis. Thus the automated maze provides data that are comparable with previous hand scored systems, but is more reliable and efficient in terms of allowing many more animals to be run at any time. This work described in Chapter 3 is currently in press in Physiology and Behavior and is co-authored by Dana Giulian and Larry Krom.

3. Increased lead ingestion can be produced in weanling rats that have been made calcium deficient, although no other manipulation attempted served to increase lead intakes. Normal rats show no preference for lead solutions, reducing percentage ingested as concentration increases. Injections of rats with lead did not reduce the amount of lead ingested voluntarily indicating that rats unlikely monitor absolute body levels of lead. Making rats iron

deficient failed to have any effect on lead ingesting, thus ruling out an alternative hypothesis that lead pica is caused by an iron deficiency. When lead injected rats were presented with calcium and water solutions, the female rats ingested a significantly greater volume of calcium than did control rats, thus indicating another behavioral interaction between lead and calcium. We have proposed a model by which calcium deficiency symptoms might be alleviated by lead ingestion. This symptom alleviation would reinforce the lead ingestion behavior the same way that calcium ingestion has been shown to be reinforcing. Any subsequent symptoms that the organism developed as a result of continued lead ingestion would not be readily associated with lead ingestion given its rewarding properties. This model is clearly quite speculative at this point given the paucity of data. A less strong conclusion from this work, however, would at least call for a careful examination of the nutritional backgrounds of low income children who are susceptible to lead pica. While there have been some studies dealing with the effects of iron deficiency and iron therapy on children with lead pica, these have been quite negative, and there has been no examination of calcium deficiency in such children. These results on rats would suggest this would be quite important to do. The results of this work as described in Chapter 4 are in press with Science.

4. In additional studies of lead pica we showed that adult rats which were calcium deficient failed to show the increased lead ingestion behavior, indicating that calcium deficiency with respect to lead pica is more potent as a phenomenon among younger organisms. We were able to again replicate the results with calcium deficiency leading to increased lead ingestion in weanling rats, but when these rats were switched to high calcium diet, their lead ingestion immediately decreased. Thus the lead pica did not continue past the restoration of calcium deficiency. Whether the lead ingestion behavior would continue beyond acute calcium deficiency if sub-minimal levels of calcium were provided has not yet been demonstrated. It will be necessary to show some persistence of the lead ingestion beyond acute calcium deficiency in order to account for the human results.

Several experiments were attempted to test our model of the acquisition of lead pica. In each case the results were inconclusive. The primary reason for our difficulty appears to be the negative taste of lead. If lead is provided as the only solution available for ingestion, there is greatly reduced food and fluid intake. This could be due to the development of lead symptoms, but could just as easily be due to the aversion of the taste of lead. Thus further work needs to be done with palatable or at least neutral lead solutions, before our hypothesis of the acquisition of lead pica can be finally tested.

5. In a related study the effects of protein malnutrition on learning was studied in rats. While there have been many studies on protein malnutrition that have been performed the literature is quite confusing about whether deficits appear or do not appear. One of the difficulties has been that investigators refer to low or moderate levels of protein deficiency with

quite different levels of protein actually being involved, that a variety of quite different behavioral tasks have been used, and that there has been no attempt to examine the effects of protein administration during each of the major developmental stages - prenatally, postnatally, post weaning, while evaluating any deficits with the same learning measures. We have started and are still in the midst of such a systematic study. We have used three levels of protein - 3%, 14%, and 25% and have administered these diets during each developmental stage according to a parametric design. All animals have been evaluated for the effects of malnutrition at the same age, using the same task - the series of twelve Hebb-Williams maze problems devised by Davenport et al. (1971). Since Davenport and Dorcey (1972) demonstrated that these tasks were the most effective indices of learning deficits, we feel they should adequately monitor learning deficits with protein deficiency. Our preliminary results have shown absolutely no effect of protein malnutrition post weaning, with somewhat more severe effects occuring as a result of prenatal rather than postnatal manipulations. We have also found more severe impairments resulting from animals fed the lowest levels of protein diets. Thus both age of manipulation and level of malnutrition can with confidence be said to be of importance, since the same procedures and the same measures of learning were used throughout.

The overall results of this work indicate that experimental work with animals can be fruitful in investigating the behavioral correlates of environmental disruptions such as malnutrition and lead exposure. We have found that both lead and protein malnutrition have their most severe effects at the earliest developmental stages, and that learning deficits - at least in the lead poisoned rats can appear when there is no longer any evidence of metabolic correlates of lead. This fact if it holds true for humans would indicate that there may well be cases of mental retardation which cannot be attributed to lead poisoning which may in fact have been induced by early lead exposure. Also since lead levels that affect the learning ability of offspring had no effect when administered to the mother indicates that perhaps human mothers exposed to sub-clinical levels of lead might be affecting their fetuses or new born infants without experiencing any symptoms themselves. Similarly the pronounced effects of protein malnutrition administered only during the prenatal period, suggest the need for adequate nutrition for human mothers from the onset of pregnancy in order to insure the normality of their children.

The findings of increased voluntary lead consumption by rats that have been made calcium deficient, indicates yet another nutritional variable that needs to be carefully examined in humans. It is reported Davis et al (1969) that of all nutrient, calcium is the one least likely to be provided to low income children in adequate amounts. If there is a causal link between calcium deficiency and lead ingestion, one would want to rapidly assure that all families regardless of income had access to adequate calcium diets for their children. With the current reduction of the school milk program, such a policy would appear to be unlikely in the near future. However, a considerably earlier period (during the first year of life) is likely to be more critical in terms of controlling the child's subsequent lead pica.

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